**Patulibacter minatonensis** gen. nov., sp. nov., a novel actinobacterium isolated using an agar medium supplemented with superoxide dismutase, and proposal of *Patulibacteraceae* fam. nov.

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A novel Gram-positive bacterial strain, designated KV-614T, was isolated from a soil sample using an agar medium supplemented with superoxide dismutase. Based on 16S rRNA gene sequence analysis, it was found that the strain represented a novel deep-rooting lineage within the class *Actinobacteria* and clustered with yet-uncultivated bacteria from terrestrial environments and some unidentified strains isolated by unique methods. The most closely related established genus was *Conexibacter* (92.4 % sequence similarity to *Conexibacter woesei* DSM 14684T). Cells of strain KV-614T were rod-shaped and motile with long flagella. The strain was catalase-positive, oxidase-negative and grew under aerobic conditions. The cell-wall peptidoglycan contained meso-diaminopimelic acid as the diagnostic diamino acid and alanine and glutamic acid. The peptidoglycan acyl type was acetyl. The only detected isoprenoid quinone was demethylmenaquinone with seven isoprene units (DMK-7). Mycolic acids were not detected. The predominant cellular fatty acid was ν9c-octadecenoic acid (C18 : 1 ν9c). Minor components were 12-methyl tetradecanoic acid (anteiso-C15 : 0) and 14-methyl hexadecanoic acid (anteiso-C17 : 0). The DNA G+C content was 72 mol%. On the basis of phenotypic and genotypic characteristics, it is proposed that strain KV-614T represents a new genus and a novel species, *Patulibacter minatonensis* gen. nov., sp. nov., in the class *Actinobacteria*. The type strain is KV-614T (= NRRL B-24346T = JCM 12834T = NBRC 100761T). The creation of the family *Patulibacteraceae* fam. nov. is proposed to encompass the genus *Patulibacter* gen. nov.

**INTRODUCTION**

The number of micro-organisms that have been successfully identified and cultured in vitro represents only a small portion of the total existing in nature; it has been estimated that the number of known bacteria is less than 10 % of the total number of bacterial species in the world (Whitman et al., 1998; Schleifer, 2004). For many unknown micro-organisms, the appropriate cultivation conditions have not yet been found. In the course of searching for factors which promote bacterial colony growth, we found that using an isolation agar medium supplemented with superoxide dismutases (SODs) increased the number of colonies isolated from a soil sample, a response that was further enhanced by the addition of catalase (Takahashi et al., 2003). In this paper, we report the taxonomic characterization and classification of strain KV-614T isolated from a soil sample using agar medium supplemented with SOD.

**METHODS**

**Isolation and cultivation conditions.** Strain KV-614T was isolated from a soil sample collected at Minato-Ku, Tokyo, Japan, using an agar medium supplemented with SOD following the method of Takahashi et al. (2003). Glucose/peptone/meat extract (GPM) agar medium, consisting of 1 % D-glucose (Wako Pure Chem. Ind.), 0.5 % peptone (Kyokuto Seiyaku Co.), 0.5 % meat extract (Kyokuto Seiyaku Co.), 0.3 % NaCl and 1.2 % agar (Wako Pure Chem. Ind.) supplemented with 30 U ml⁻¹ *Escherichia coli* SOD (Sigma), was used for strain isolation. The strain was cultured on 1/5-strength nutrient agar (1/5 NA; Difco), ISP 3 medium (Shirling & Gottlieb, 1966), heart infusion agar, R2A agar and Todd–Hewitt agar (Difco) and yeast extract/glucose agar (YD agar; containing 1.0 % yeast

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**ABBREVIATIONS:** DAP, diaminopimelic acid; SOD, superoxide dismutase.

The GenBank/EMBL/DDBJ accession number for the partial 16S rRNA gene sequence of strain KV-614T is AB193261.
extract (Difco), 1-0% glucose and 1-2% agar) for 7 days at 27 °C. Trypticase soy broth (BBL) was used for liquid culture. The strain was stored at 5 °C under lyophilization.

**Morphology.** The morphological characteristics of the strain were observed using a transmission electron microscope (JEM-1200EXIII; JEOL) after incubation for 3 days at 27 °C in trypticase soy broth. Negative staining of cells was performed with 1% uranyl acetate. Gram-staining was performed using a Gram's stain reagent kit (Nacalai Tesque).

**Physiology.** Substrate utilization patterns were studied by using a GP2 microplate from the Biolog identification system. Enzyme activities were examined by using API ZYM test strips (bioMérieux). The API 20 NE test system (bioMérieux) was used for the investigation of additional physiological characteristics. Cells were grown on 1/5 NA for these tests. For determination of the temperature range for growth, bacteria were cultured on 1/5 NA medium. NaCl tolerance was examined using YD agar medium. The pH growth range was also determined using YD agar that was adjusted to pH values ranging between 5 and 9 with HCl or NaOH. Antibiotic susceptibility was determined by placing antibiotic discs (KB Disk; Eiken) on 1/5 NA plates seeded with suspensions of strain KV-614T.

**Chemotaxonomic characterization.** Purified cell-wall preparations were obtained using the method of Schleifer & Kandler (1972). The amino acid composition of cell-wall hydrolysates (Becker et al., 1965) was identified by TLC (Hasegawa et al., 1983). The N-acyl type of muramic acid was determined using the colorimetric method of Uchida & Aida (1977). Isoprenoid quinone was extracted as described by Collins et al. (1977). The sample was analysed by HPLC with 802-SC chromatography (JASCO) using a CAPCELL PAK C18 column (Shiseido) (Tamaoka et al., 1983) and was further analysed by MS and NMR. The detection of mycolic acids was performed by TLC (Tomiyasu, 1982). Methyl esters of cellular fatty acids were prepared by direct transmethylation with methanolic hydrochloride using cells grown on trypticase soy agar at 27 °C for 4 days and analysed on a GLC system (HP 6890; Hewlett Packard). Identification and quantification of the fatty acid methyl esters, as well as the numerical analysis of the fatty acid profiles, were performed according to the instructions for the Microbial Identification System (MIDI).

**DNA G+C content.** Chromosomal DNA was prepared following the procedure of Marmur (1961) and the DNA G+C content was determined by reverse-phase HPLC according to Tamaoka & Komagata (1984).

**Phylogenetic analysis.** DNA was isolated following the method of Marmur (1961). The 16S rDNA gene was amplified using primers described by Takahashi et al. (2002). Amplifications were performed in a TaKaRa thermal cycler (Takara) with an initial incubation of 1 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 1-5 min at 72 °C, followed by a 2 min final extension at 72 °C. The PCR products were purified using a QIAquick gel extraction kit (Qiagen) and were sequenced directly on a DNA sequencer (ABI PRISM 3100; Applied Biosystems) using PRISM ready reaction dye primer cycle sequencing kits (Applied Biosystems), according to the manufacturer’s instructions. The 16S rDNA gene sequence was manually aligned with the corresponding sequences of representative strains and clones retrieved from the DDBJ database. CLUSTAL W (Thompson et al., 1994) was used to estimate evolutionary distances (the K̄ value of Kimura, 1980) and similarity values were used to construct the phylogenetic tree by the neighbour-joining method (Saitou & Nei, 1987). The topology of the tree was evaluated by performing a bootstrap analysis (Felsenstein, 1985) using 1000 resamplings. The phylogenetic tree produced by the maximum-likelihood method was generated using PAUP* version 4.0b8 (Swofford, 2001).

**RESULTS AND DISCUSSION**

**Cultural and morphological characteristics**

Strain KV-614T forms flat and nearly transparent colonies with a whitish colour on 1/5 NA, ISP 3 medium and R2A agar and with a pale-yellow colour on heart infusion agar and Todd–Hewitt agar. Bacterial cells are Gram-positive and rod-shaped (1-2-1-5 × 0-6-0-7 μm). Cells are motile due to the presence of long flagella (Fig. 1).

**Phylogenetic analysis**

The almost-complete 16S rRNA gene sequence (1528 nt) was determined for strain KV-614T. Preliminary sequence comparisons with 16S rRNA gene sequences deposited in GenBank indicated that strain KV-614T belonged to the class Actinobacteria. A phylogenetic tree constructed using the 16S rRNA gene sequences of strain KV-614T and those of other recognized species is shown in Fig. 3. Strain KV-614T represents a novel lineage within the order Rubrobacterales of the
Actinobacteria (Stackebrandt et al., 1997), adjacent to the genera Conexibacter (Monciardini et al., 2003) and Solirubrobacter (Singleton et al., 2003). The 16S rRNA gene sequence similarity of strain KV-614T to Conexibacter woesei DSM 14684T is 92.4% and it shows 89.7% similarity to that of Solirubrobacter pauli B33D1T. The tree constructed by

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strain KV-614T and its closest clonal and cultured relatives. Clones prefixed 'TM' were from a peat bog (Rheims et al., 1996, 1999) and strains prefixed 'Ellin' were from Australian soils (Sait et al., 2002; Joseph et al., 2003). The sequences of clones YNPFFP1 and 1959-7 were available from public databases. Numbers at nodes are bootstrap values based on 1000 resamplings; only values higher than 500 are shown. The tree was rooted with Bacillus subtilis. Bar, 20 inferred nucleotide substitutions per 1000 nucleotides.

Fig. 3. Phylogenetic tree showing the position of strain KV-614T based on 16S rRNA gene sequences. Numbers at nodes indicate the level of bootstrap support based on neighbour-joining analysis of 1000 resampled datasets. Only values higher than 700 are shown. The tree was rooted with Escherichia coli. Bar, 10 inferred nucleotide substitutions per 1000 nucleotides.
maximum-likelihood method supported this result (data not shown).

**Chemotaxonomic characteristics**

To characterize strain KV-614\( ^T \) further, bacterial cell chemical constituents were analysed. Cell-wall peptidoglycan contained meso-diaminopimelic acid (DAP) as the diagnostic diamino acid and alanine and glutamic acid. The peptidoglycan appeared to be of the A1\( _{7} \) type (based on meso-DAP, direct cross-linkage). The peptidoglycan was of the acetyl type. The isoprenoid quinone was demethylmenaquinin with seven isoprene units (DMK-7), which was detected as the only component by HPLC. DMK-7 has also been found in *Enterococcus faecalis* (Hiraishi, 1988), *Pasteurella* and *Haemophilus* (Kroppenstedt & Mannheim, 1989); however, this unique isoprenoid quinone has been not reported previously for established genera of the class *Actinobacteria*. Mycolic acids were not detected. The predominant cellular fatty acid components were \( \omega_9 \)-octadecenoic acid (oleic acid, C18:1\( \omega_9 \)C, 63·2%), 12-methyl tetradecanoic acid (anteiso-C15:0, 9·8%); 14-methyl hexadecanoic acid (anteiso-C17:0, 7·7%); hexadecanoic acid (C16:0, 4·5%) and octadecanoic acid (C18:0, 4·3%). There was no match to any entry in the TDBA50 MIS library, further supporting the classification of strain KV-614\( ^T \) as a member of a novel genus and species. The DNA G+C content of strain KV-614\( ^T \) was 72 mol%.

**Physiological characteristics**

Strain KV-614\( ^T \) was aerobic, catalase-positive, oxidase-negative and able to reduce nitrate to nitrite. It did not grow on media containing \( \geq 2 \) % (w/v) NaCl. The pH range for growth was 6–8. The temperature range for growth was 16–28°C and the optimum temperature for growth was 24–27°C.

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**Taxonomic conclusions**

On the basis of phylogenetic analysis, strain KV-614\( ^T \) belongs to the order *Rubrobacterales* of the subclass *Rubrobacteridae* (Stackebrandt et al., 1997). An A residue at position 906 (E. coli sequence; Brosius et al., 1978) and a U residue at position 955, characteristics of members of the subclass *Rubrobacteridae*, were confirmed to be present in the 16S rRNA gene sequence of strain KV-614\( ^T \). The characteristic pattern of 16S rRNA gene sequence signature nucleotides of the subclass *Rubrobacteridae* (Stackebrandt, 2004), 127–234 (G–C), 291–309 (U–A), 955–1225 (U–A), 1115–1185 (C–G) and 1410–1490 (A–U), was also present.

Table 1 shows the phenotypic characteristics of strain KV-614\( ^T \) and members of two phylogenetically related genera, *Conexibacter* and *Solirubrobacter*. Like strain KV-614\( ^T \), *C. woesei* has meso-DAP as the diagnostic diamino acid in the cell-wall peptidoglycan and also produces long flagella, but it clearly differs in the major menaquinone MK-7(H\( _4 \)) and predominant fatty acids. *S. pauli* is distinguished from strain KV-614\( ^T \) in that it has non-motile cells and a different fatty acid content. Data on isoprenoid quinones and cell-wall peptidoglycan amino acids are not yet available for this genus. On the basis of phylogenetic analysis and phenotypic characteristics, we propose that strain KV-614\( ^T \) represents a novel genus and species, *Patulibacter minatonensis* gen. nov., sp. nov. (type strain KV-614\( ^T \)=NRRL B-24346\( ^T \)=JCM 12834\( ^T \)=NBRC 100761\( ^T \)).

At present, the order *Rubrobacterales* (Stackebrandt et al., 1997) contains four families, *Rubrobacteraceae*, *Conexibacteraceae*, *Solirubrobacteraceae* and *Thermoleophilaceae* (Stackebrandt, 2004). Based on the distinct phylogenetic position of *Patulibacter minatonensis* gen. nov., sp. nov. within the order *Rubrobacterales* and the differences observed in the pattern of 16S rRNA gene sequence signature nucleotides, a new family, *Patulibacteraceae* fam. nov., is proposed.

**Table 1. Differential characteristics of strain KV-614\( ^T \) and members of related genera**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of rods (( \mu )m)</td>
<td>1·2–1·5</td>
<td>0·9–1·2</td>
<td>1·4</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile, long flagella</td>
<td>Motile, long flagella</td>
<td>Non-motile</td>
</tr>
<tr>
<td>Optimum growth temperature (°C)</td>
<td>24–27</td>
<td>28–37</td>
<td>28–30</td>
</tr>
<tr>
<td>pH range</td>
<td>6–8</td>
<td>7–7·5</td>
<td>6–7·7</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Major fatty acids (&gt;8%)</td>
<td>C18:1( \omega_9 )C (63%); a-C15:0 (10%); a-C17:0 (8%)</td>
<td>C18:1( \omega_9 )C (41%); i-C16:0 (16%); C17:1( \omega_9 )c (14%); C16:0 (13%)</td>
<td>i-C16:0 (54%); C18:1( \omega_9 )c (36%)</td>
</tr>
<tr>
<td>Diagnostic amino acid in peptidoglycan</td>
<td>meso-DAP</td>
<td>meso-DAP</td>
<td>ND</td>
</tr>
<tr>
<td>Isoprenoid quinone</td>
<td>DMK-7</td>
<td>MK-7(H( _4 ))</td>
<td>ND</td>
</tr>
</tbody>
</table>

Strains: 1, KV-614\( ^T \); 2, *Conexibacter woesei* ID 131577\( ^T \) (data from Monciardini et al., 2003); 3, *Solirubrobacter pauli* B33D1\( ^T \) (Singleton et al., 2003). ND, Not determined; –, negative; +, positive.
The fatty acid profile is dominated by C18 : 1 bacterium with spreading growth). *Patulibacter* mas. n. contains material respiration is aerobic. Cell-wall peptidoglycan contains meso-DAP as the diagnostic diamino acid and alanine and glutamic acid. The peptidoglycan is of the acetyl type. The fatty acid profile is dominated by C18 : 1 (ω9c). Mycolic acids are absent. The predominant isoprenoid quinone is DMK-7. The DNA G+C content is 72 mol%. The type species is *Patulibacter minatonensis* sp. nov.

**Description of *Patulibacter minatonensis* sp. nov.**

*Patulibacter minatonensis* (mi.na.to.nen sis. N.L. masc. adj. minatonensis pertaining to Minato-ku, the ward of Tokyo, Japan, where the type strain was isolated).

Exhibits the following properties in addition to those given in the genus description. Forms flat and nearly transparent colonies with a whitish or pale yellow colour. Cells are non-endospore-forming rods. Bacterial respiration is aerobic. Cell-wall peptidoglycan contains meso-DAP as the diagnostic diamino acid and alanine and glutamic acid. The peptidoglycan is of the acetyl type. The fatty acid profile is dominated by C18 : 1 (ω9c). Mycolic acids are absent. The predominant isoprenoid quinone is DMK-7. The DNA G+C content is 72 mol%. The type species is *Patulibacter minatonensis* sp. nov.

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